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Formation of D-3-hydroxybutyryl-coenzyme A by an acetoacetyl-coenzyme A reductase in *Syntrophomonas wolfei* subsp. *wolfei*

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Abstract. Cell-free extracts of *Syntrophomonas wolfei* subsp. *wolfei* synthesized D-(−)-3-hydroxybutyryl-coenzyme A (CoA) (the stereoisomer required for the synthesis of poly-β-hydroxyalkanoate) from acetoacetyl-CoA, but not crotonyl-CoA, and NAD(P)H. Ammonium sulfate fractionation and ion exchange chromatography separated an acetoacetyl-CoA reductase activity that formed D-(−)-3-hydroxybutyryl-CoA from the β-oxidation enzyme activity, L-(+)-3-hydroxyacyl-CoA dehydrogenase. The former activity was further purified by hydroxylapatite and affinity chromatography. The most pure acetoacetyl-CoA reductase preparations formed D-(−)-3-hydroxybutyryl-CoA from acetoacetyl-CoA and had high specific activities using either NADH or NADPH as the electron donor. Thus, *S. wolfei* makes D-(−)-3-hydroxybutyryl-CoA by an acetoacetyl-CoA reductase rather than by a D-isomer specific enoyl-CoA hydratase and the reducing equivalents required for PHA synthesis from acetoacetyl-CoA can be supplied from the NADH made during β-oxidation.

Key words: Poly-β-hydroxylalkanoate – Methanogenesis – Syntrophic – Fatty acid metabolism – *Syntrophomonas wolfei*

The intracellular polyester, poly-3-hydroxyalkanoate (PHA) serves as a carbon storage material in many different bacteria (Anderson and Dawes 1990; Dawes and Senior 1973). The final step in PHA synthesis is the incorporation of D-(−)-3-hydroxyacyl-CoA into the polymer by PHA synthetase. It has been shown that at least two different pathways exist for the synthesis of D-(−)-3-hydroxybutyryl-CoA. One of these is the pathway found in *Rhodospirillum rubrum* (Moskowitz and Merrick 1969) in which acetoacetyl-CoA is first reduced to L-(+)-3-hydroxybutyryl-CoA by an NAD-dependent dehydrogenase. This intermediate is then converted to D-(−)-3-hydroxybutyryl-CoA in two successive steps. First, an L-specific enoyl-CoA dehydratase converts L-(+)-3-hydroxybutyryl-CoA into crotonyl-CoA and then, D-(−)-3-hydroxybutyryl-CoA hydratase converts crotonyl-CoA into D-(−)-3-hydroxybutyryl-CoA. These two activities function in the reverse direction in mitochondria to convert D-(−)-3-hydroxyacyl-CoA intermediates formed from *cis*-unsaturated fatty acids into the L-isomer required for β-oxidation (Smeland et al. 1989). The other pathway for PHA synthesis is that found in *Azotobacter beijerinckii* (Ritchie et al. 1971; Senior and Dawes 1973), *Alcaligenes eutrophus* (Haywood et al. 1988; Oeding and Schlegel 1973), and *Zoogloea ramigera* (Saito et al. 1977) in which acetoacetyl-CoA is converted directly into D-(−)-3-hydroxybutyryl-CoA by an NADPH-linked acetoacetyl-CoA reductase.

In *Syntrophomonas wolfei*, PHA is synthesized by two routes, the condensation and subsequent reduction of two acetyl-CoA molecules and by the incorporation of a β-oxidation intermediate without cleavage of a C−C bond (Amos and McInerney 1989). Enzymatically, *S. wolfei* could use a pathway similar to that found in *R. rubrum* (Moskowitz and Merrick 1969) where D-(−)-3-hydroxybutyryl-CoA is made directly from crotonyl-CoA generated in β-oxidation or from 2 acetyl-CoA molecules by operating the β-oxidation pathway in the reverse direction. The other possibility is that *S. wolfei* makes D-(−)-3-hydroxybutyryl-CoA from acetoacetyl-CoA generated by β-oxidation or the condensation of 2 acetyl-CoA molecules using an acetoacetyl-CoA reductase as found in aerobic bacteria (Haywood et al. 1988; Ritchie et al. 1971; Saito et al. 1977). Wofford et al. (1986) showed that *S. wolfei* has high activities of the β-oxidation enzymes. However, it is not known whether *S. wolfei* has enzymatic activities to form D-(−)-3-hydroxybutyryl-CoA required for PHA synthesis from crotonyl-CoA or from acetoacetyl-CoA.

In this paper, we show that *S. wolfei* has an acetoacetyl-CoA reductase activity that forms D-(−)-3-hydroxybutyryl-CoA from acetoacetyl-CoA using either NADH or NADPH.

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Materials and methods

Organism and growth conditions

Syntrophomonas wolfei subsp. *wolfei* was grown in pure culture in the basal medium of McInerney et al. (1979) except that the rumen fluid concentration was 2% rather than 5% (vol/vol) and 25 mM sodium crotonate rather than sodium butyrate was the energy source. Ten-liter cultures were prepared as described previously (Wofford et al. 1986). These large cultures were inoculated with 100 ml of a mid-exponential phase culture of *S. wolfei*. The cells were harvested aerobically at 4 °C using a CEPA IIigh Speed Separator (Carl Padberg, Lahr/Schwarzwald, FRG) equipped with a clarifying cylinder. The cell paste was resuspended in 50 mM sodium phosphate buffer (pH 7.5) and the cells were washed twice by centrifugation (10000 × *g*, 10 min, 4 °C) using a fixed-angle centrifuge and resuspending the cells in this buffer. The final cell pellet was dispensed into cryovials (approximately 3 g of cells each) and stored in liquid nitrogen.

Preparation of the crude extract

Thawed cells were resuspended in breaking buffer and the cells were broken by two passages through a French pressure cell at 12,000 psi. The breaking buffer contained 10 mM each of potassium phosphate (pH 7.5), β-mercaptoethanol, and ethylenediamine tetraacetic acid (EDTA), disodium salt, and 10% (vol/vol) glycerol. Unbroken cells and other debris were removed by centrifugation (10000 × g, 10 min, 4 °C).

Enzyme assays

Each activity was corrected for the amount of activity observed in the absence of substrate and was linear with respect to time and protein concentration. No activity was observed when boiled enzyme preparations were used.

Acetoacetyl-CoA reductase activity was measured in both directions (Ritchie et al. 1971). The reduction of acetoacetyl-CoA was measured using a reaction mixture (final volume of 0.66 ml) that contained 60 mM potassium phosphate (pH 5.5), 12 mM $MgCl_2 \cdot 6\,H_2O$, 0.5 mM DL-dithiothreitol (DTT), 0.24 mM NAD(P)H and 0.3 mM acetoacetyl-CoA. The enzyme preparation was incubated in the reaction mixture without acetoacetyl-CoA for 5 min before the addition of the pyridine nucleotide. The rate of pyridine nucleotide oxidation was determined spectrophotometrically at 340 nm in the absence and presence of acetoacetyl-CoA. The oxidation of DL-3-hydroxybutyryl-CoA was assayed using a reaction mixture (final volume 0.66 ml) that contained 100 mM glycine-NaOH buffer (pH 9.0), 12 mM $MgCl_2 \cdot 6\,H_2O$, 0.5 mM DTT, 0.24 mM $NAD(P)^+$, and 0.3 mM DL-3-hydroxybutyryl-CoA. The reaction was performed as described above except that the formation of NAD(P)H was measured.

Enoyl-COA hydratase activity was measured using the direct procedure of Fong and Schulz (1981) and by a modification of the indirect method of Wofford et al. (1986). For the direct method, the decrease in absorbance caused by the reduction of the double bond in crotonyl-CoA was measured at 263 nm using a molar extinction coefficient of 6700 $m^{-1}\,cm^{-1}$. For the indirect method, the assay mixture (1.0 ml) contained 100 mM potassium phosphate (pH 8.0), 100 mM KCl, 0.1 mg of bovine serum albumin per ml, 0.4 mM NAD, 30 µM crotonyl-CoA and 2 units of L-(+)-3-hydroxylacyl-CoA dehydrogenase. The NAD-dependent oxidation of L-(+)-3-hydroxybutyryl-CoA produced by enoyl-CoA hydratase activity was followed by measuring the production of NADH at 340 nm.

L-3-hydroxyacyl-CoA dehydrogenase activity and 3-ketoacyl-CoA thiolase activity were measured as described previously (Wofford et al., 1986).

D-(−)-3-hydroxybutyryl-CoA formation

The formation of the D-isomer of 3-hydroxybutyryl-CoA from acetoacetyl-CoA by crude extracts and enzyme preparations of *S. wolfei* was determined enzymatically after removing the CoA group. The reaction mixture was the same as that used to assay acetoacetyl-CoA reductase activity except the concentrations of NADPH and acetoacetyl-CoA were increased to 0.58 mM and to 0.54 mM, respectively. The acetoacetyl-CoA dependent oxidation of NADPH was followed until no further oxidation of NADPH was observed. Then 60 µl of 3.5 M NaOH was added and the reaction mixture was boiled for 10 min to remove the CoA group. The solution was centrifuged (12000 × g, 3 min, room temperature) and the amount of D-(−)-3-hydroxybutyrate present was determined spectrophotometrically using a commercially available enzyme kit (Sigma Chemicals, St. Louis, MO., USA). D-(−)-3-Hydroxybutyrate (Sigma) served as the positive control and L-(+)-3-hydroxybutyrate served as the negative control. L-(+)-3-Hydroxybutyrate was prepared by hydrolyzing L-(+)-3-hydroxybutyryl-CoA (Sigma) as described above.

Partial purification of the acetoacetyl-CoA reductase activity

All procedures were performed at 4 °C unless otherwise indicated.

Ammonium sulfate fractionation

The crude extract (11.5 ml) was brought to 30% saturation with ammonium sulfate (anhydrous). The solution was stirred slowly for 1 h and centrifuged (10000 × *g*, 10 min). Enough solid ammonium sulfate was added to the supernate to obtain 80% saturation. After stirring for 1 h, the precipitate was collected by centrifugation and resuspended in a minimal volume of breaking buffer. The suspended material was dialyzed overnight against 1 liter of breaking buffer.

Ion exchange chromatography

The dialyzed enzyme preparation from the previous step was applied to a column (2.5 cm × 10 cm) of DEAE Bio-Gel A (Bio-Rad Laboratories, Richmond, Calif., USA) which had been equilibrated with 500 ml of breaking buffer. After the protein was applied to the column, the column was washed with breaking buffer until no protein was detected in the effluent. The enzyme activity was eluted in two steps, first using 500 ml of breaking buffer with 50 mM KCl followed by the same volume of breaking buffer with 150 mM KCl. Five-ml fractions were collected and assayed for enoyl-CoA hydratase activity (direct and indirect methods) and acetoacetyl-CoA reductase activity (NADPH oxidation method). Fractions that eluted with breaking buffer containing 50 mM KCl were assayed for L-(+)-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities. Fractions with either enoyl-CoA hydratase activity or acetoacetyl-CoA reductase activity were tested for the ability to form D-(−)-3-hydroxybutyryl-CoA. The fractions with the highest acetoacetyl-CoA reductase activity and that formed D-(−)-3-hydroxybutyryl-CoA were pooled and diazyled for 4 h against 1 l of 5 mM potasssium phosphate buffer (pH 7.5) with 10% (vol/vol) glycerol, 10 mM β-Mercaptoethanol, and 10 mM EDTA.

Hydroxylapatite chromatography

The dialyzed fraction from above was applied to a column (2 cm × 6 cm) of hydroxylapatite which had been equilibrated with 500 ml of the buffer used above for dialysis. The activity was eluted with a gradient (220 ml) of potassium phosphate (5 mM to 200 mM) (pH 7.5) in this buffer.

Affinity chromatography

The fractions with acetoacetyl-CoA reductase activity and that formed D-(−)-3-hydroxybutyryl-CoA obtained by hydroxylapatite chromatography were pooled and applied to a column (1.5 cm × 7 cm) of Reactive Red 120-Agarose (Sigma) equilibrated with 500 ml of 30 mM potassium phosphate buffer (pH 7.5) with 10% (vol/vol) glycerol, 10 mM β-mercaptoethanol, and 10 mM EDTA. The enzyme activity was eluted using a gradient (500 ml) of KCl from 30 mM to 3 M in the buffer used to equilibrate the column. Fractions containing acetoacetyl-CoA reductase activity were pooled and dialyzed overnight against 2 l of breaking buffer. The dialyzed enzyme solution was concentrated for 8 h with Aquacide I (Calbiochem, La Jolla, Calif., USA). The concentrated sample was then dialyzed overnight with 2 liters of breaking buffer. The concentration of glycerol in the dialyzed preparation was increased to 50% (vol/vol) and the preparation was then stored in liquid nitrogen.

Protein determination

The amount of protein present in fractions obtained after column chromatography was estimated using the absorbance ratio A_{280}/A_{260} (Hanson and Phillips 1981). Otherwise, the amount of protein was determined colorimetrically using the bicinchoninic acid method (Smith et al. 1985).

Electrophoresis

Native gel electrophoresis of a sample (48 µg of protein) of the pooled fraction obtained after affinity chromatography was performed as described previously (Orr et al. 1972).

Results and discussion

D-(−)-3-hydroxybutyryl-CoA formation

The crude extract had high levels of enoyl-CoA hydratase activity when assayed by the direct or indirect method (Table 1). However, the formation of D-(−)-3-hydroxybutyryl-CoA was observed only when the indirect method was used. In the indirect method, L-(+)-3-hydroxybutyryl-CoA dehydrogenase is added to the reaction mixture to convert the product of the enoyl-CoA hydratase reaction, L-(+)-3-hydroxybutyryl-CoA, to acetoacetyl-CoA. This suggested that crotonyl-CoA must be converted to acetoacetyl-CoA before D-(−)-3-hydroxybutyryl-CoA can be formed. To test this possibility, the ability of *Syntrophomonas wolfei* crude extracts to form D-(−)-3-hydroxybutyryl-CoA from acetoacetyl-CoA was determined. Crude extracts of *S. wolfei* had high levels of acetoacetyl-CoA reductase activity and formed D-(−)-3-hydroxybutyryl-CoA from acetoacetyl-CoA. If NAD(P)H or acetoacetyl-CoA was omitted from the reaction mixture, no D-isomer was formed (data not shown). These experiments suggested that *S. wolfei* has a nicotinamide nucleotide-dependent acetoacetyl-CoA reductase activity that forms D-(−)-3-hydroxybutyryl-CoA as found in aerobic bacteria (Haywood et al. 1988; Ploux et al. 1988, Ritchie et al. 1971; Saito et al. 1977) rather than a D-enoyl-CoA hydratase as was previously suggested (Amos and McInerney 1989).

Table 1. Formation of D-(−)-3-hydroxybutyryl-CoA by crude extracts of *Syntrophomonas wolfei*

Assay method	Specific activity[a] (µmol min^{-1} mg^{-1})	D-(−)-3-hydroxybutyryl-CoA (µmol)[b]
Enoyl-CoA hydratase		
direct	50.5	<0.01
indirect	27	0.21
Acetoacetyl-CoA reductase	69	0.12

[a] The protein concentration of the crude extract was 0.5 to 0.8 mg/ml
[b] The amount of D-(−)-3-hydroxybutyryl-CoA formed from 0.5 µmol of acetoacetyl-CoA after no further NAD(P)H oxidation was observed

Purification of the acetoacetyl-CoA reductase activity

The acetoacetyl-CoA reductase activity was partially purified in order to determine whether the enzyme activity responsible for the formation of the D-isomer of 3-hydroxybutyryl-CoA was the same or different from the activity that metabolizes the L-isomer of 3-hydroxybutyryl-CoA produced during β-oxidation. The activity of enoyl-CoA hydratase was also monitored to determine if this activity was involved in the formation of D-(−)-3-hydroxybutyryl-CoA.

The pellet from the first ammonium sulfate precipitation (30%) had very little acetoacetyl-CoA reductase or enoyl-CoA hydratase activities. However, the precipitate obtained after the ammonium sulfate concentration reached 80% saturation contained high levels of both of these activities (data not shown).

Ion exchange chromatography separated the acetoacetyl-CoA reductase activity into two fractions, one which eluted after the KCl concentration was increased to 50 mM, and the other which eluted after the KCl concentration was increased to 150 mM (Fig. 1 and Table 2). Most of the acetoacetyl-CoA reductase eluted when the concentration of KCl was increased to 50 mM.

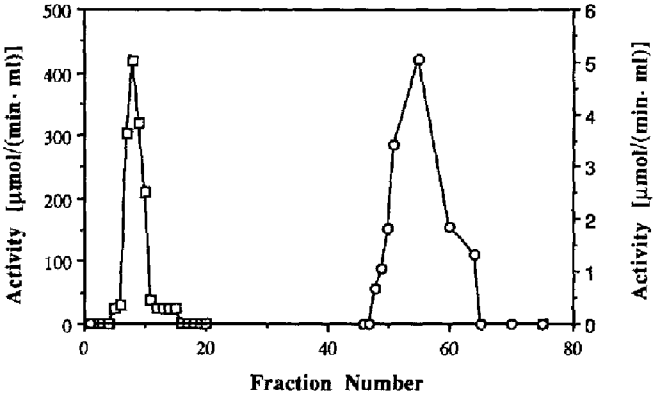

Fig. 1. Elution of the acetoacetyl-CoA reductase activities from DEAE BIO-GEL A column. Fractions 1 to 40 *(squares)* were obtained after elution using a buffer containing 50 mM KCl; fractions 41 to 80 *(circles)* were obtained using a buffer with 150 mM KCl. The righthand *y-axis* scale refers to activities found in peaks 41 to 80

Table 2. Purification of acetoacetyl-CoA reductase activity from *S. wolfei*

Purification step	Total protein (mg)	Specific activity (μmol min^{-1} mg^{-1})	D-(−)-3-hydroxybutyryl-CoA formed (μmol)
Crude extract	637	–[a]	0.12
$(NH_4)_2SO_4$ (30–80%)	229	–	ND[b]
DEAE Bio-Gel A			
50 mM KCl	ND	–	<0.01
150 mM KCl	30.7	1.24	0.37
Hydroxylapatite	30.0	1.25	0.37
Reactive Red 120-Agarose	3.4	47.0	0.43

[a] Due to the competing activity of the NADPH-oxidizing β-oxidation enzyme in the crude extract, it was not possible to calculate the specific activity for this enzyme at this stage of the purification
[b] ND, not determined

Table 3. Specific activity and D-(−)-3-hydroxybutyryl-CoA formation of the affinity-purified acetoacetyl-CoA reductase with different nicotinamide adenine dinucleotides[a]

Addition	Specific activity (μmol min^{-1} mg^{-1})	D-(−)-3- hydroxybutyryl-CoA (μmol)
NAD(P)H	<0.04	ND
NADPH + AcAcCoA[b]	4.82	0.41
NADH + AcAcCoA	10.6	0.38

[a] Pooled and concentrated fractions of acetoacetyl-CoA reductase obtained after affinity chromatography were assayed by the reduction of acetoacetyl-CoA using the reaction mixture containing potassium phosphate, MgCl, and dithiothreitol as described in the 'Material and methods' with the indicated additions listed above
[b] AcAcCoA, acetoacetyl-CoA

The peak fractions of acetoacetyl-CoA reductase activity that eluted with 50 mM KCl also had high levels of the three β-oxidation enzymes, enoyl-CoA hydratase (4.1 U/ml) L-(+)-3-hydroxyacyl-CoA dehydrogenase (1.7 U/ml) and 3-ketoacyl-CoA thiolase activities (2 U/ml). These fractions were pooled and tested for the ability to form D-(−)-3-hydroxybutyryl-CoA from acetoacetyl-CoA. No formation of this compound was detected. However, these fractions formed L-(+)-3-hydroxybutyryl-CoA as evidenced by the high levels of activity found for enoyl-CoA hydratase using the indirect method which measures the amount of L-(+)-3-hydroxybutyryl-CoA formed from crotonyl-CoA by a coupled enzyme assay. The reaction mixtures used for the indirect assay of enoyl-CoA hydratase were also checked for the formation D-(−)-3-hydroxybutyryl-CoA; again, this compound was not formed. This provided additional evidence that enoyl-CoA hydratase activity did not produce D-(−)-3-hydroxybutyryl-CoA from crotonyl-CoA.

The second peak of acetoacetyl-CoA reductase activity eluted when the KCl concentration was increased to 150 mM and the pooled fractions from this peak formed D-(−)-3-hydroxybutyryl-CoA. These fractions did not contain detectable levels of enoyl-CoA hydratase or 3-ketoacyl-CoA thiolase activities (data not shown). These results show that *S. wolfei* has an enzyme activity, different from the β-oxidation enzymes, which reduces acetoacetyl-CoA to D-(−)-3-hydroxybutyryl-CoA.

The D-(−)-3-hydroxybutyryl-CoA-forming acetoacetyl-CoA reductase activity was further purified by using hydroxylapatite and affinity chromatography. Because of the competing, NADH-oxidizing, β-oxidation enzyme activities, it is not possible to determine the specific activity of this enzyme in the crude extract or to calculate the overall fold purification. The hydroxylapatite chromatography step gave no net purification of the activity. However, the affinity chromatography step resulted in a 37-fold increase in the specific activity (Table 2). The acetoacetyl-CoA reductase activity eluted from the affinity chromatography column between 300 and 400 mM KCl (data not shown) rather than at much higher salt concentrations (3 M) as found with the activity from *Zoogloea ramigera* (Fukui et al., 1987). Native gel electrophoresis showed that the partially purified enzyme obtained after affinity chromatography contained two major protein bands (data not shown).

Characteristics of acetoacetyl-CoA reductase activity

The partially pure enzyme had very high specific activities for the reduction of acetoacetyl-CoA using NADH or NADPH (Table 3), and did not exhibit any activity for the oxidation of DL-3-hydroxybutyryl-CoA (data not shown). The lack of oxidation of DL-3-hydroxybutyryl-CoA and the relatively high rate of acetoacetyl-CoA reduction indicates that the primary purpose of this enzyme in *S. wolfei* is the reduction of acetoacetyl-CoA. However, since the substrate was a mixture of the D- and L-isomers, it may be possible that the L-isomer was a strong inhibitor of the enzyme. The acetoacetyl-CoA reductase that forms D-(−)-3-hydroxybutyryl-CoA in *Z. ramigera* is specific for NADPH and has an optimal pH of 8.1 (Saito et al. 1977; Ploux et al. 1988). The acetoacetyl-CoA reductases from *Azotobacter beijerinckii* and *Alcaligenes eutrophus* preferentially use NADPH, but show appreciable activity with NADH (20% of that detected with NADPH) (Ritchie et al. 1971; Haywood et al. 1988). The acetoacetyl-CoA reductase activity (D-isomer-forming) from *S. wolfei* uses either NADH or NADPH (Table 3) as the electron donor and no activity was detected at pH of 8.0 using either a Tris · HCl or a phosphate buffer (data not shown). Thus, this enzyme more closely resembles that found in *A. beijerinckii* and *A. eutrophus*.

Previous work showed that *S. wolfei* synthesizes PHA by two routes, directly from a β-oxidation intermediate without cleavage of a carbon-carbon bond and from the condensation and subsequent reduction of two acetyl-CoA molecules (Amos and McInerney 1989, 1991). Acetoacetyl-CoA can be made by either route and, once formed, it can be converted to D-(−)-3-hydroxybutyryl-CoA by the acetoacetyl-CoA reductase activity. Since

the only reaction that generates reducing power for the cell with crotonate as the energy source is that catalyzed by L-(+)-3-hydroxyacyl-CoA dehydrogenase, the nature of the electron donor for PHA synthesis was an important question. This enzyme is usually specific for NAD (Nunn 1986). Although the cofactor specificity of this activity in *S. wolfei* is not known, high levels of activity were detected when NADH was used as the electron donor (Fig. 1; Wofford et al. 1986). The finding that the acetoacetyl-CoA reductase involved in the synthesis of the D-isomer of 3-hydroxybutyryl-CoA in *S. wolfei* uses either nicotinamide nucleotide shows that the NADH produced from the oxidation of L-(−)-3-hydroxyacyl-CoA during β-oxidation can be used directly for PHA synthesis. Thus, a mechanism to interconvert NADH and NADPH by transhydrogenase activity or by some other route is not required.

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